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1 *Short title:* Volatiles, pseudomonads, *Agaricus bisporus* primordia

2 **Volatile C8 compounds and pseudomonads influence primordium**  
3 **formation of *Agaricus bisporus***

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12 **Abstract:** Primordium formation of *Agaricus bisporus* depends on the presence of a ‘casing  
13 layer’ containing stimulatory bacteria, and on sufficient air exchange. The influence of  
14 specific pseudomonad populations and volatile organic compounds (VOCs) on primordium  
15 formation of *A. bisporus* was studied in microcosm cultures. VOCs produced by *A. bisporus*  
16 mycelium were predominantly C8 compounds, some of which could inhibit primordium  
17 formation, with 1-octen-3-ol being most inhibitory. A VOC produced by the rye grain  
18 substrate on which *A. bisporus* was grown, 2-ethyl-1-hexanol, also inhibited primordium  
19 formation. 2-Ethyl-1-hexanol and 1-octen-3-ol were metabolized by pseudomonad  
20 populations and adsorbed by activated charcoal, with both modes of removal enabling  
21 primordium formation in the casing. Removal of VOCs by ventilation also enabled  
22 primordium formation to occur under axenic conditions. The presence of 2-ethyl-1-hexanol  
23 and 1-octen-3-ol in the microcosms resulted in higher total bacterial and pseudomonad  
24 populations in the casing. The stimulatory effects of the casing and its microbiota, and air  
25 exchange on primordium formation of *A. bisporus* are due, at least partly, to the removal of  
26 inhibitory C8 compounds produced by the mycelium and its substrate. Monitoring and

1 controlling the levels of these inhibitory VOCs in mushroom culture should enable  
2 primordium formation of *A. bisporus* to be more efficiently and precisely controlled.

3 **Key words:** casing, 2-ethyl-1-hexanol, mushroom, 1-octen-3-ol, *Pseudomonas putida*

#### 4 INTRODUCTION

5 Fructification in the button mushroom (*Agaricus bisporus*) begins with the aggregation of  
6 mycelium into thick strands, followed by the formation of primordia, some of which develop  
7 further into sporophores. This is a highly inefficient process, with generally less than 7 % of  
8 initiated primordia developing further into sporophores (Flegg 1979; Noble et al 2003).

9 Primordium formation is stimulated by a reduction in temperature, and an increase in  
10 ventilation, which is considered to have an effect through reduced CO<sub>2</sub> concentration  
11 (Tschierpe & Sinden 1964; Flegg & Wood 1985). *A. bisporus* also has a requirement for a  
12 ‘casing layer’ (casing) which has imprecisely defined chemical and microbiological properties  
13 that stimulate primordium formation (Wood 1976; Flegg 1979). The presence of stimulatory  
14 bacteria in the casing, of which *Pseudomonas putida* is regarded as most significant (Hayes et  
15 al 1969; Rainey et al 1990), is usually necessary for primordium formation to occur.

16 Activated charcoal and other adsorbent materials can obviate the requirement for stimulatory  
17 pseudomonads (Eger 1961; Long & Jacobs 1974; Noble et al 2003), possibly by adsorbing  
18 compounds that are inhibitory to primordium formation. Attempts to isolate putative  
19 inhibitors to primordium formation from activated charcoal casing have been confounded by  
20 the large number of compounds adsorbed and identified (Grove 1981). However, several  
21 volatile C<sub>8</sub> compounds were identified that were characteristic of *A. bisporus* and of  
22 sufficiently low molecular weight to be readily adsorbed by activated charcoal. Of these, 1-  
23 octen-3-ol was found to inhibit primordium formation *in vitro* on malt agar without inhibiting  
24 mycelial growth (Wood & Blight 1982).

25 Since the function of the casing and mechanism of primordium formation are not  
26 fully understood, fructification in button mushroom culture has relied on the empirical  
27 selection of casing materials (Noble et al 2003; Beyer 2004) and by reducing temperature and  
28 CO<sub>2</sub> concentration at an appropriate stage (Flegg & Wood 1985). Attempts to reduce the

1 stimulation of excess numbers of primordia and improve the efficiency of fructification, by  
2 manipulation of temperature or ventilation, or selection of less conducive casing materials,  
3 have resulted in excessive vegetative mycelial growth in the casing ('stroma') and/or reduced  
4 sporophore production (Flegg 1979; Flegg & Wood 1985).

5 This work aimed to improve the understanding of the mechanism of primordium  
6 formation of *A. bisporus in vivo* by: (i) determining the stimulatory effect of isolates of *P.*  
7 *putida* and related *Pseudomonas* species defined by their 16S rRNA sequences (Elomari et al  
8 1996; Anzai et al 2000); (ii) measuring the effect of the casing microbiota on the  
9 concentration of specific VOCs produced by mushroom mycelium and its substrate; and (iii)  
10 determining the effect of these VOCs on primordium formation and on the populations of  
11 bacteria in the casing.

## 12 MATERIALS AND METHODS

13 *Axenic microcosm tests.*—A 500 mL glass microcosm culture system (Noble et al 2003) was  
14 used to examine growth in axenic casing materials. The microcosms were filled with a 10 mm  
15 layer (30 g) of sterile rye grain 'spawn', which served as a nutritional substrate, colonized with  
16 the *A. bisporus* strain A15 (Sylvan Spawn, Peterborough, UK), except where stated. This  
17 substrate was covered with 60 g (about 17 mm layer) of casing, consisting of a mixture of  
18 black peat and CaCO<sub>3</sub> (4:1 v/v) or activated charcoal (granular, 4–8, Sigma Aldrich, Poole,  
19 Dorset, UK). Casing material was autoclaved at 121 C for 2 h for axenic treatments.  
20 Temperatures (25 C for 7 d followed by 16 C), CO<sub>2</sub> concentrations (>0.5%v/v followed by  
21 0.08–0.12%v/v) and casing matric potential (–1 to –2 kPa) in the microcosms were  
22 maintained as described in Noble et al (2003). Microcosms were arranged in an incubator in a  
23 randomized block design with each incubator shelf containing a complete replicate block of  
24 each set of treatments. The numbers of primordia > 1 mm diameter were recorded 28 d after  
25 the microcosms were filled, i.e. 12 to 16 d after the first primordia had reach this size.  
26 Bacterial numbers in the casing were recorded before and after mushroom culture as  
27 described below.

1 *Bacterial isolates and populations in casing materials.* —Seven isolates of *P. putida* or  
2 closely related species were obtained from casing materials and other growing media (Fermor  
3 *et al.* 2000): *P. veronii* (MAR2 and MAR12), *P. poae* (n12,), *P. putida* (T2/6 and T1/4) and  
4 an unnamed species *P. PsI* (NSC4 and NSC6). Three further isolates of *P. putida* (4Alux,  
5 Paw8 and Paw340) were obtained from the Warwick HRI culture collection. The taxonomy of  
6 these isolates was determined by comparison of the 16 S rRNA gene sequences with  
7 sequences that have been obtained from related *Pseudomonas* isolates in the EMBL database,  
8 using the program FASTA (Genetics Computer Group, University of Wisconsin) (TABLE I).

9       The *Pseudomonas* isolates were grown in nutrient broth (LB broth (Miller) Merck,  
10 Darmstadt, Germany). The bacteria were harvested by centrifugation, re-suspended in sterile  
11 distilled water to  $10^9$  cfu mL<sup>-1</sup>. Bacterial suspensions (1 mL) were then inoculated on to  
12 axenic peat + CaCO<sub>3</sub> casing in *A. bisporus* microcosm cultures. Axenic and non-axenic casing  
13 were used as controls. There were three replicate microcosms of each *Pseudomonas* isolate  
14 and axenic or non-axenic culture treatment. The experiment was repeated four times.

15       Bacterial populations in the casing in microcosms were determined by preparing  
16 suspensions of 1 g casing in 9 mL of sterile Ringers solution (Fisher Scientific,  
17 Loughborough, UK). Serial dilutions of the suspension were then plated on nutrient agar  
18 (CM309, Oxoid, Basingstoke, UK) and pseudomonas isolation agar (PIA) (Difco  
19 Laboratories, Detroit, USA) and then incubated at 25 C for 48 h to determine the total  
20 bacterial populations as colony forming units per g casing (cfu g<sup>-1</sup>), and to estimate the  
21 proportion which were pseudomonads.

22 *Analysis of volatile organic compounds in microcosms.*—Two methods involving gas  
23 chromatography-mass spectrometry (GC-MS) were used for analyzing the VOCs produced by  
24 *A. bisporus* mycelium in the casing and in the headspace of axenic and non-axenic  
25 microcosms. A solvent extraction method involved washing of casing in diethyl ether  
26 followed by low temperature analysis of the solvent. Casing (5 g) was put in the porous  
27 thimble of a Soxhlet apparatus and VOCs were extracted in 45 mL of diethyl ether for one

1 hour. The resulting samples (1  $\mu\text{L}$ ) were then injected on to a GC column (SPB1,  $30 \times 0.25$   
2 mm,  $0.25 \mu\text{m}$  film thickness, Supelco, Bellefonte, PA, USA) of a Shimadzu QP 5000 GC-MS  
3 system (Shimadzu UK, Milton Keynes). The flow rate of the eluting gas, helium, was  $1.1 \text{ mL}$   
4  $\text{min}^{-1}$ . The temperature ramp of the GC oven was set to: rate: temperature (C): time (s)  
5 (0:50:5, 20:150:0 and 4:250:5).

6           Although unsuitable for analysis of high moisture content casing, a thermal  
7 desorption method was used for analyzing VOCs in the headspace of microcosms, due to  
8 higher resolution than the solvent extraction method. VOCs from the microcosm headspace  
9 were firstly pre-concentrated on to adsorbents (Carbotrap 20/40 mesh and Tenax TA 60/80,  
10 Supelco, 0.3 g) contained within 6 mL porcelain crucibles placed on the surface of the casing.  
11 The concentrated VOCs were then thermally desorbed from 220 mg samples of the  
12 adsorbents into a GC-MS system for identification and quantification. A Hewlett Packard (hp,  
13 Stockport, Cheshire, UK) GC-MS system consisting of a 5890 II Series gas chromatograph  
14 and a 5972A mass selective detector (MSD II) was used for analysis. Chromatographic  
15 retention time and mass spectral matching were used to confirm VOC identity. A 25 m fused  
16 silica (cross linked methyl siloxane) hp-1 column with an internal diameter of 0.2 mm and a  
17  $0.34 \mu\text{m}$  film with a 1 m, 'Q plot', deactivated fused silica guard column (internal diameter  
18  $0.53 \text{ mm}$ ), containing a porous polymer of divinylbenzene (Supelco) was used. The flow rate  
19 of the eluting gas, helium, was  $0.75 \text{ mL min}^{-1}$ . An Optic temperature programmable injector  
20 (Ai Cambridge, Pampisford, Cambridge, UK) was used to desorb headspace samples from the  
21 adsorbents and was initially set to  $30 \text{ C}$  and then heated at  $16 \text{ C s}^{-1}$  to  $250 \text{ C}$ . An electronic  
22 pressure controller was used to offset peak pressure broadening with increasing GC column  
23 temperature. The GC oven conditions were an initial temperature of  $40 \text{ C}$ , then increased to  
24  $220 \text{ C}$  at  $15 \text{ C min}^{-1}$  and remaining at  $220 \text{ C}$  for 1 min. The GC-MS interface was at  $280 \text{ C}$ .  
25 The mass spectrometer scanned from 35 to 250 mass units every 0.2 s to give responses in the  
26 ng range.

1 VOCs detected by the mass spectrometers were identified using a probability based  
2 matching algorithm and a NIST mass spectral library (National Institute of Standards and  
3 Technology, Gaithersburg, MD, USA). Compounds were declared unknown if their matching  
4 probability was less than 80 (100 being a perfect match).

5 Samples of adsorbent or casing were analyzed using the solvent extraction and  
6 thermal desorption methods respectively, before use for *A. bisporus* culture to test for  
7 background VOCs. VOC levels obtained showed evidence of a mean-variance relationship.  
8 These concentrations were subjected to a logarithmic transformation prior to analysis.  
9 *Primordium formation and production of VOCs by A. bisporus strains.* —Microcosms were  
10 prepared using axenic and non-axenic peat + CaCO<sub>3</sub> casing as previously described. The *A.*  
11 *bisporus* strains A15 and 5776 (Le Lion, Varrains, France) were selected on the basis of  
12 known difference in primordium formation in culture. Microcosms without *A. bisporus*  
13 inoculum were prepared to test for background VOCs in the culture. Crucibles containing the  
14 Carbotrap or Tenax adsorbents were placed in each microcosm after 7 d, at the time of  
15 transfer from 25 C to 16 C. The adsorbents were analyzed by the thermal desorption method  
16 after a further 21 d, at the same of recording numbers of primordia and casing bacteria. Eight  
17 replicate microcosms were prepared for each *A. bisporus* inoculum, casing sterility and  
18 adsorbent treatments, giving a total of 96 microcosms.

19 *Effect of C8 compounds on primordium formation and casing bacteria.* —The effect of  
20 introducing specific VOCs identified from the above experiment into the atmosphere of  
21 microcosms was examined. Microcosms were prepared using axenic activated charcoal and  
22 non-axenic peat + CaCO<sub>3</sub> casing as previously described. The following VOCs (10 µL) were  
23 placed in 20 ml glass beakers in microcosms, after 7 and 17 d: 2-ethyl-1-hexanol, octane, 1-  
24 octanol, 3-octanone, *trans*-3-octene and 1-octen-3-ol. 2-Ethyl-1-hexanol was also used at 1  
25 µL per beaker. Beakers with water were used as a control. Samples of the casing materials  
26 were analyzed by the solvent extraction method. Three replicate microcosms of each VOC  
27 and casing material treatment were prepared, giving a total of 48 microcosms.

1 *Effect of VOCs on primordium formation and casing bacteria in ventilated flasks.* —  
2 Composted substrate (Noble et al 1998) (100 g) was filled into 350 mL glass jars. After  
3 autoclaving (axenic jars only), the substrate was inoculated at 2% w/w with rye grain spawn  
4 of the *A. bisporus* strain Sylvan A15. The jars were incubated under sterile conditions for 14 d  
5 at 25 C. The colonized substrate was then covered with 80 g of the above axenic or non-  
6 axenic peat + CaCO<sub>3</sub> casing and the jars placed in 10 L 'Quickfit' multiadapter flasks (Fisher  
7 Scientific, Loughborough, UK) with 5 jars per flask. Sterile water to a depth of 30 mm in the  
8 base on the flasks maintained a relative humidity > 90%. The flasks were then sealed with  
9 bacterial air vents and incubated at 25 C for about 8 d until mycelium became visible at the  
10 surface of the casing, which was then watered with 10 mL sterile water on each jar. The flasks  
11 were then transferred to a room at 18 C and connected to a humidified, filtered air flow of 15  
12 L h<sup>-1</sup> to maintain a CO<sub>2</sub> concentration of 0.7–0.11% v/v.

13           The air flow to the multiadapter flasks was passed through sealed 300 mL flasks  
14 containing 3–5 mL of ethanol, 2-ethyl-1-hexanol, octane, 3-octanone, 1-octen-3-ol or *cis*-3-  
15 octen-1-ol. The level of VOCs was maintained by replenishment at 4–5 d intervals and by  
16 placing the 300 mL flasks in ice to reduce volatilization. Axenic and non-axenic cultures  
17 aerated without VOCs were used as controls. Two replicate multiadapter flasks aerated with  
18 each VOC, and four replicate axenic and non-axenic controls were prepared. The numbers of  
19 primordia, sporophores (developmental stage 4, Hammond & Nichols 1976), total bacteria  
20 and pseudomonads in the casing were recorded 20 d after the casing was applied as  
21 previously described. The supply of VOCs to flasks was then removed and the flasks aerated  
22 with fresh air only. The number of primordia and sporophores was then recorded after a  
23 further 10 d.

24           Bacterial and primordial populations showed evidence of a mean-variance  
25 relationship. These populations were respectively subjected to a logarithmic or square root  
26 transformation prior to analysis. All differences in the results section were significant at  $P <$   
27 0.05, or if stated,  $P < 0.01$  or 0.001.



## 1 RESULTS

2 *Effect of Pseudomonas isolates on primordium formation.*—The *Pseudomonas* isolates added  
3 to axenic casing were found to differ in terms of growth in the casing and in stimulating  
4 primordium initiation (FIG.1). Isolates n12 and NSC4 stimulated the formation of more  
5 primordia than other isolates whereas the *P. veronii* isolates MAR2 and MAR12 stimulated  
6 the formation of the fewest primordia, although had the highest pseudomonad populations in  
7 the casing at the end of the experiment ( $3.5 \times 10^8$  and  $3.7 \times 10^8$  cfu g<sup>-1</sup> respectively compared  
8 with  $1.4 \times 10^8$  –  $2.6 \times 10^8$  cfu g<sup>-1</sup> for the other isolates). None of the *Pseudomonas* isolates  
9 added to axenic casing stimulated the formation of as many primordia as non-axenic casing  
10 (FIG. 1) ( $P < 0.001$ ). In non-axenic casing during *A. bisporus* culture, there were slight  
11 increases in the populations of pseudomonads, from  $6.0 \times 10^5$  to  $7.6 \times 10^5$  cfu g<sup>-1</sup>, and in total  
12 bacteria, from  $5.1 \times 10^6$  to  $6.0 \times 10^6$  cfu g<sup>-1</sup>. However, the final population of pseudomonads  
13 in non-axenic casing was significantly ( $P < 0.001$ ) smaller than the final populations in the  
14 axenic casing inoculated with *Pseudomonas* isolates.

15 *Primordium formation and production of VOCs by A. bisporus strains.*—Significant  
16 formation of primordia only occurred in the non-axenic microcosms, with the *A. bisporus*  
17 strain A15 forming more primordia than the strain 5776 (TABLE II) ( $P < 0.01$ ). By the time of  
18 primordia assessment, one or two primordia had developed further into immature sporophores  
19 (stages 1 and 2, Hammond & Nichols 1976) in some of the microcosms. However, the  
20 formation and development of primordia and sporophores was slower in the microcosms than  
21 in the larger scale flask culture due to the lower temperature (16 C compared with 18C)  
22 (Flegg 1979; Noble et al 2003).

23 In the presence of *A. bisporus* inoculum, the pseudomonad population of non-axenic  
24 casing increased from  $7.5 \times 10^5$  to  $8.1 \times 10^5$  cfu g<sup>-1</sup>, but declined to  $1.2 \times 10^4$  cfu g<sup>-1</sup> without  
25 *A. bisporus* inoculum. The total bacterial population of the casing declined from  $1.9 \times 10^7$  cfu  
26 g<sup>-1</sup> to  $6.8 \times 10^6$  and  $2.5 \times 10^5$  cfu g<sup>-1</sup> in the presence and absence of *A. bisporus* inoculum

1 respectively. Final populations of total bacteria and pseudomonads in the casing of non-  
2 axenic microcosms were unaffected by the *A. bisporus* strain and adsorbent treatments.

3 No VOCs were detected in the adsorbents before use. Over 30 VOCs were identified in  
4 the chromatograms of the adsorbent traps from microcosms, with concentrations of most  
5 VOCs significantly higher in the Tenax than in the Carbotrap. A number of compounds with  
6 molecular weights exceeding 150 were detected in trace amounts (e.g. 3,7-dimethyl-1,6-  
7 octadien-3-ol); these are excluded from TABLES II and III. Several VOCs were detected in  
8 both the microcosms with and without *A. bisporus* inoculum, with 2-ethyl-1-hexanol, benzyl  
9 alcohol and benzene compounds being most abundant (TABLE III). For all of these VOCs, the  
10 concentrations were highest in the axenic, uninoculated microcosms and were reduced by the  
11 presence of the microbiota in the non-axenic microcosms, and the *A. bisporus* inoculum. The  
12 exception was 1-bromo-heptane, which was greater in the microcosms containing *A. bisporus*  
13 ( $P < 0.001$ ). 1-Bromo-octane was only detected in microcosms inoculated with *A. bisporus*  
14 (TABLE II). These halogenated VOCs may be *A. bisporus* metabolites of 1-bromo-decane,  
15 which was most abundant in the axenic microcosms ( $P < 0.001$ ). VOCs that were present only  
16 in the microcosms inoculated with *A. bisporus* were exclusively 8-carbon compounds (TABLE  
17 II). With the exception of 1-octanol, the concentrations were higher in the axenic microcosms  
18 than in the non-axenic microcosms.

19 *Effect of 8-carbon compounds on primordium formation and casing bacteria.*—The 8-carbon  
20 compounds in the microcosms did not affect mycelial growth in the casing. The numbers of  
21 primordia that formed on axenic activated charcoal and non-axenic peat +  $\text{CaCO}_3$  casing in  
22 the presence of different C8 compounds is shown in FIG. 2. The axenic charcoal casing  
23 resulted in more primordia than the non-axenic peat +  $\text{CaCO}_3$  casing ( $P < 0.01$ ). The presence  
24 of 2-ethyl-1-hexanol in the microcosms suppressed primordium formation. In the microcosms  
25 containing peat +  $\text{CaCO}_3$  casing, 1  $\mu\text{L}$  of 2-ethyl-1-hexanol was sufficient to cause a  
26 suppressive effect; in the microcosms containing axenic activated charcoal, 10  $\mu\text{L}$  were  
27 required. The other C8 compounds did not significantly affect primordium formation in the  
28 microcosms.

1 No VOCs were detected in the casing materials before use. At the end of the  
2 experiment, no C8 compounds were detected using the solvent method of extraction in any of  
3 the microcosms containing peat + CaCO<sub>3</sub> casing or in the control microcosms containing  
4 axenic activated charcoal (FIG. 2). At the end of the experiment, 2-ethyl-1-hexanol, 1-octen-3-  
5 ol, 3-octanone and *trans*-3-octene were detected in the activated charcoal casing at similar  
6 concentrations.

7 The total bacterial population of the control treatment casing did not change  
8 significantly during the culture period ( $2.5 \times 10^7$  and  $2.3 \times 10^7$  cfu g<sup>-1</sup> at the beginning and  
9 end respectively), whereas the pseudomonad population increased from  $7.5 \times 10^5$  cfu g<sup>-1</sup> to  
10  $1.1 \times 10^6$  cfu g<sup>-1</sup>. Final total bacterial numbers in the peat + CaCO<sub>3</sub> casing were higher in the  
11 presence of 1-octen-3-ol and 2-ethyl-1-hexanol than in the control microcosms and in the  
12 presence of the other C8 compounds (FIG. 3). The pseudomonad population was increased by  
13 the presence of 2-ethyl-1-hexanol in the microcosms (P<0.01) (FIG. 3).

14 *Effect of VOCs on primordium formation and casing bacteria in ventilated flasks.* —The  
15 presence of ethanol or C8 compounds into ventilated flask culture reduced mycelial growth in  
16 the casing. Primordia and sporophores were produced in both axenic and non-axenic  
17 ventilated flask culture, although the numbers of sporophores were greater in non-axenic  
18 conditions (TABLE IV). The presence of ethanol or 1-octen-3-ol almost completely inhibited  
19 primordium formation, which was also suppressed by 2-ethyl-1-hexanol and *cis*-3-octen-1-ol.  
20 Octane suppressed the numbers of primordia but not of sporophores and 3-octanone had no  
21 significant effect (TABLE IV). Following the removal of the VOCs from the air stream into  
22 the flasks, primordium formation occurred within 7 d and at least one sporophore per jar was  
23 produced in all the treatments.

24 The pseudomonad population of the casing in the control treatment remained stable  
25 during the *A. bisporus* culture period although the total numbers of bacteria declined (TABLE  
26 IV). 2-Ethyl-1-hexanol and 1-octen-3-ol increased the total bacterial population of the casing

1 during the culture period ( $P < 0.001$ ) and all the VOCs, except 3-octanone, increased the  
2 population of pseudomonads in the casing.

### 3 DISCUSSION

4 This work has confirmed earlier research (Grove 1981; Combet et al 2006) that VOCs  
5 produced by *A. bisporus* mycelium are predominantly C8 compounds. The *in vivo* results  
6 from microcosm cultures support the hypothesis based on *in vitro* agar plate cultures (Wood  
7 & Blight 1982), that some of these C8 compounds can inhibit primordium formation, with 1-  
8 octen-3-ol being most inhibitory. The results have also shown that a VOC produced by the rye  
9 grain substrate on which *A. bisporus* is grown, 2-ethyl-1-hexanol, can inhibit primordium  
10 formation. 2-Ethyl-1-hexanol is a metabolite known to be produced by *Acremonium*  
11 *obclavatum* and *Aspergillus versicolor* (Enzeonu et al 1994; Pasanen et al 1997) although its  
12 source in the substrate in these experiments was not established. 2-Ethyl-1-hexanol and 1-  
13 octen-3-ol were metabolized by the casing microbiota and adsorbed by activated charcoal  
14 casing, with both modes of removal enabling primordium formation, as previously suggested  
15 by Eger (1972) and Long & Jacobs (1974). 2-Ethyl-1-hexanol was inhibitory to primordium  
16 formation at lower concentrations than C8 compounds produced by *A. bisporus* and at  
17 concentrations below which a negative effect on mycelial growth was observed. The  
18 inhibitory effect was less with activated charcoal casing than with peat-based casing, probably  
19 due to the greater adsorption capacity of activated charcoal compared with peat. Wood &  
20 Blight (1982) noted that 1-octen-3-ol inhibited fruitbody formation but not mycelial growth  
21 on agar culture. Mau et al. (1992) identified a compound 10-oxo-*trans*-8-decenoic acid  
22 (ODA) from *A. bisporus* sporophores that stimulated mycelial growth and stipe elongation of  
23 *A. bisporus*. Supplementation of the casing with ODA resulted in an increase in the number of  
24 sporophores produced and they suggested that ODA may be involved in the initiation of  
25 fruiting, although this was not examined.

26 The presence of 2-ethyl-1-hexanol and 1-octen-3-ol resulted in higher total bacterial  
27 and pseudomonad populations in the casing. These bacteria were therefore able to utilize and  
28 metabolize the inhibitory VOCs, as previously suggested by Hayes et al (1969) and Eger

1 (1972). It is well established that pseudomonads are able to utilize C8 compounds as  
2 substrates (Chakrabarty et al 1973). In these experiments, the population of pseudomonads in  
3 the casing tended to increase during *A. bisporus* culture, whereas the total bacterial population  
4 remained stable or slightly declined. The increasing dominance of pseudomonads in the  
5 casing bacterial population during *A. bisporus* culture was also observed by Hayes & Nair  
6 (1974) and indicates the significance of pseudomonads in metabolizing VOCs produced by *A.*  
7 *bisporus* mycelium. However, none of the *Pseudomonas* isolates tested were able stimulate  
8 primordia formation in axenic casing to the same extent as a naturally occurring microbiota in  
9 non-axenic casing, in spite of the inoculated casing having a significantly higher  
10 pseudomonad population. This confirms earlier work by Eger (1972) and indicates that either  
11 a mixed pseudomonad or bacterial population is more stimulatory to primordium formation  
12 than individual pseudomonads. An isolate of *P. poae* stimulated the formation of more  
13 primordia than several *P. putida* isolates and two isolates of *P. veronii* had little or no  
14 stimulatory effect on primordium formation. This confirms previous work which has  
15 identified *P. putida* and closely related species as being stimulatory to primordium formation,  
16 with several other *Pseudomonas* species, including *P. aeruginosa*, *P. agarici*, *P. fluorescens*,  
17 *P. reactans* and *P. tolaasii*, being non-stimulatory (Hayes et al 1969; Rainey et al 1990;  
18 Fermor et al 2000). Significant differences in the stimulatory behaviour of several *P. putida*  
19 isolates were observed, in agreement with Fermor et al (2000) but not Rainey et al (1990).  
20 Further work is needed to test if the VOC metabolizing capability of individual *Pseudomonas*  
21 isolates, or of mixed bacterial populations, relates to their stimulatory effect on primordium  
22 formation.

23 Removal of inhibitory VOCs by ventilation enabled primordium formation to occur on  
24 peat-based casing under axenic conditions. This has not been previously observed, probably  
25 due to the enclosure and inadequate ventilation of axenic culture systems that have been used  
26 before (Long & Jacobs 1974; Yeo 1980; Noble et al 2003). The requirement for sufficient air  
27 exchange for primordium formation of *A. bisporus* has been attributed solely to a reduction in  
28 CO<sub>2</sub> concentration (Tschierpe & Sinden 1964; Flegg & Wood 1985). In the present

1 microcosm experiments, a reduction in CO<sub>2</sub> concentration using soda lime adsorbent did not  
2 overcome the inhibitory effect of the VOCs. Experimentation is needed to separate the  
3 independent effects of inhibitory VOCs and CO<sub>2</sub> on primordium formation.

4 This work has shown that the stimulatory effects of the casing and its microbiota, and  
5 air exchange on primordium formation of *A. bisporus* are due, at least partly, to the removal  
6 of inhibitory C8 compounds produced by the mycelium and its substrate. Mushroom strains  
7 differ in their production of C8 compounds which influence their primordium formation.  
8 Monitoring and controlling the levels of these inhibitory VOCs in mushroom culture should  
9 enable primordium formation of *A. bisporus* to be more efficiently and precisely controlled.  
10 This could be achieved by intermittent adsorbent trapping and subsequent analysis of VOCs  
11 from culture room air (Pfeil & Mumma 1992), and adjustment of room ventilation  
12 accordingly. The influence of different mushroom strains and substrates on the production of  
13 C8 compounds should also be investigated further.

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#### 16 REFERENCES

17 Anzai Y, Kim H, Park J-Y, Wakabayashi H, Oyaizu H, 2000. Phylogenetic affiliation of the  
18 pseudomonads based on the 16S rRNA sequence. *International Journal of Systematic and*  
19 *Evolutionary Microbiology* 50: 1563–1589.

20

21 Beyer DM, 2004. Polishing up and managing your casing. *Mushroom News* 52(10):10-21.

22

23 Chakrabarty AM, Chou G, Gunsalus IC, 1973. Genetic Regulation of Octane Dissimilation  
24 Plasmid in *Pseudomonas*. *Proceedings of the National Academy of Sciences* 70: 1137–1140.

25

26 Combet E, Henderson J, Eastwood DC, Burton KS, 2006. Eight carbon volatiles in  
27 mushrooms and fungi: properties, analysis and biosynthesis. *Mycoscience* 47:317–326.

28

- 1 Eger G, 1961. Untersuchungen über die Function der Deckschicht bei der  
2 Fruchtkörperbildung des Kulturchampignons, *Psalliota bispora* Lange. Archiv für  
3 Mikrobiologie 39: 313–334.
- 4  
5 —, 1972. Experiments and comments on the action of bacteria on sporophore initiation in  
6 *Agaricus bisporus*. Mushroom Science 8: 719–725.
- 7  
8 Elomari M, Coroler L, Hoste B, Gillis M, Izard D, Leclerc H, 1996. DNA relatedness among  
9 *Pseudomonas* strains isolated from natural mineral waters and proposal of *P. veronii* sp. nov.  
10 International Journal of Systematic Bacteriology 46: 1138–1144.
- 11  
12 Enzeonu IM, Price DL, Simmons RB, Crow SL, Ahearn DG, 1994. Fungal production of  
13 volatiles during growth on fibreglass. Applied and Environmental Microbiology 60: 4172–  
14 4173.
- 15  
16 Fermor TR, Lincoln S, Noble R, Dobrovin-Pennington A, 2000. Microbiological  
17 properties of casing. In: van Griensven LJLD, ed. Science and cultivation of edible fungi.  
18 Rotterdam: Balkema. p 447–454.
- 19  
20 Flegg PB, 1979. Effect of temperature on sporophore initiation and development in *Agaricus*  
21 *bisporus*. Mushroom Science 10: 595–602.
- 22  
23 —, Wood DA, 1985. Growth and fruiting. In: Flegg PB, Spencer DM, Wood DA, eds. The  
24 biology and technology of the cultivated mushroom. Chichester, UK: John Wiley & Son. p  
25 141–178.
- 26  
27 Grove JF, 1981. Volatile compounds from the mycelium of the mushroom *Agaricus bisporus*.  
28 Phytochemistry 20: 2021–2022.
- 29

- 1 Hammond JBW, Nichols R, 1976. Carbohydrate metabolism in *Agaricus bisporus* (Lange)  
2 Sing.: changes in soluble carbohydrates during growth of mycelium and sporophore. Journal  
3 of General Microbiology 93:309–320.  
4
- 5 Hayes WA, Nair NG, 1974. Effects of volatile metabolic by-products of mushroom mycelium  
6 on the ecology of the casing layer. Mushroom Science 9(I): 259–268.  
7
- 8 —, Randle PE, Last FT, 1969. The nature of the microbial stimulus affecting sporophore  
9 formation in *Agaricus bisporus* (Lange) Sing. Annals of Applied Biology 64: 177–187.  
10
- 11 Long PE, Jacobs L, 1974. Aseptic fruiting of the cultivated mushroom *Agaricus bisporus*.  
12 Transactions of the British Mycological Society 63: 99–107.  
13
- 14 Mau J-L, Beelman RB, Ziegler GR, 1992. Effect of 10-oxo-*trans*-decenoic acid on growth of  
15 *Agaricus bisporus*. Phytochemistry 31: 4059–4064.
- 16 Noble R, Gaze RH, Willoughby N, 1998. A high yielding substrate for mushroom  
17 experiments: formula 3. Mushroom Journal 587: 27–28.
- 18  
19 —, Fermor TR, Lincoln S, Dobrovin-Pennington A, Mead A, Li R, Evered C, 2003.  
20 Primordia initiation of mushroom (*Agaricus bisporus*) strains on axenic casing materials.  
21 Mycologia 95: 620–629.
- 22  
23 Pasanen P, Korpi A, Kalliokoski P, Pasanen A-L, 1997. Growth and volatile metabolite  
24 production of *Aspergillus versicolor* in house dust. Environment International 23: 425–432.  
25
- 26 Pfeil RM, Mumma RO, 1992. Air sampling of volatiles from *Agaricus bisporus* in a  
27 mushroom facility and from mushroom compost. HortScience 27: 416–419.  
28



- 1 Rainey PB, Cole ALJ, Fermor TR, Wood DA, 1990. A model system for examining  
 2 involvement of bacteria in basidiome initiation of *Agaricus bisporus*. Mycological Research  
 3 94: 191–194.
- 4
- 5 Tschierpe H, Sinden JW, 1964. Weitere Untersuchungen über die Bedeutung von  
 6 Kohlendioxyd für die Fruktifikation des Kulturchampignons *Agaricus campestris* var.  
 7 *bisporus* (L.) Lge. Archiv für Mikrobiologie 49: 405–425.
- 8
- 9 Wood DA, 1976. Primordium formation in axenic cultures of *Agaricus bisporus* (Lange)  
 10 Sing. Journal of General Microbiology 95: 313–323.
- 11  
 12 —, Blight M, 1982. Sporophore initiation in axenic culture. Report of the Glasshouse  
 13 Crops Research Institute for 1981. p 140.
- 14
- 15 Yeo SG, 1980. The ecology of paper mill by-product and its evaluation as a casing medium in  
 16 the culture of *Agaricus bisporus* (Lange) Pilat. [Doctoral Dissertation]. Birmingham, UK:  
 17 University of Aston. 344 p.

#### 19 FIGURE CAPTIONS

20 FIG. 1. Numbers of primordia > 1 mm diam (square root transformed values) the formed on  
 21 non-axenic peat + CaCO<sub>3</sub> casing (control, □) and on axenic casing inoculated with different  
 22 *Pseudomonas* isolates (■). No primordia formed on uninoculated sterile casing. Length of  
 23 vertical bar indicates least significant difference between treatments ( $P = 0.05$ , 121 df).

24

25 FIG. 2. Effect of different C8 compounds (10 µL unless stated) in the atmosphere of  
 26 microcosms on the numbers of primordia > 1 mm diam (square root transformed values) that  
 27 formed on non-axenic peat + CaCO<sub>3</sub> casing (□) and axenic charcoal casing (■). Length of  
 28 vertical bar indicates least significant difference between treatments ( $P = 0.05$ , 32 df). Figures

1 above bars are the concentrations of the respective C8 compound detected in the casing at the  
2 end of the experiment, where measured ( $\mu\text{g g}^{-1}$ ).

3

4 FIG. 3. Effect of different C8 compounds (10  $\mu\text{L}$  unless stated) in the atmosphere of  
5 microcosms on the populations of total bacteria ( $\square$ ) and pseudomonads ( $\blacksquare$ ) in non-axenic peat  
6 +  $\text{CaCO}_3$  casing. Length of vertical bar indicates least significant difference between  
7 treatments ( $P = 0.05$ , 32 df).

8

9 FOOTNOTE

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TABLE I. Similarity of the 16S rRNA gene sequence of *Pseudomonas* isolates to the best matches in the EMBL database using the program FASTA.

<i>Pseudomonas</i> isolate	Species name with greatest homology and EMBL accession no.	Sequence Length	Similarity (%)
n12	<i>P. poae</i> DSM14936T	1529	100.0
NSC4	<i>P. PsI</i> AF105387	1407	98.6
NSC6	<i>P. PsI</i> AF105387	1407	97.7
4Alux	<i>P. putida</i> D85994	1259	96.5
T1/4	<i>P. putida</i> D85994	1259	100.0
T2/6	<i>P. putida</i> D85994	1259	98.7
Paw8	<i>P. putida</i> KT2440	302614	99.0
Paw340	<i>P. putida</i> KT2440	302614	99.0
MAR2	<i>P. veronii</i> CFML 92-134	1430	97.6
MAR12	<i>P. veronii</i> CFML 92-104	1430	97.6

TABLE II. Volatile organic compounds (VOCs) detected only in axenic and non-axenic microcosms containing *Agaricus bisporus* inoculum, and numbers of primordia > 1 mm diam. Values for primordia and VOC concentrations in adsorbents are respectively square root and logarithmic transformed means of microcosms containing Carbotrap and Tenax adsorbents, with back-transformations shown in parentheses.

Sterility	Axenic	Non-axenic	Axenic	Non-axenic	LSD( $P=0.05$ )
<i>A. bisporus</i> strain	A15	A15	5776	5776	69 df
primordia per microcosm	0.68 (0.1)	7.06 (49.4)	0.61 (0.0)	2.1 (4.0)	0.42
VOC ( $\mu\text{g g}^{-1}$ )					
1-bromo-octane	1.19 (3.29)	-0.10 (0.89)	1.83 (6.22)	0.55 (1.72)	0.88
1,6-octadien-3-ol	1.98 (7.21)	-2.63 (0.06)	1.83 (6.24)	-1.17 (0.30)	1.06
octane	-0.13 (0.87)	-3.41 (0.02)	0.00 (0.99)	-0.90 (0.40)	1.40
1-octanol	-3.19 (0.03)	-2.86 (0.05)	-2.81 (0.05)	-3.30 (0.03)	0.73
3-octanone	3.15 (23.21)	-2.39 (0.08)	3.49 (32.91)	2.17 (8.71)	0.94
1-octen-3-ol	2.13 (8.38)	-1.80 (0.16)	2.19 (8.93)	1.30 (3.65)	1.65

TABLE III. Volatile organic compounds (VOCs) detected in the highest concentrations in axenic and non-axenic microcosms, both with and without *Agaricus bisporus*. Values are logarithmic transformations of mean VOC concentrations in Carbotrap and Tenax adsorbents and of *A. bisporus* strains A15 and 5776, with back-transformations shown in parentheses.

Sterility	Axenic	Non-axenic	Axenic	Non-axenic	LSD( $P=0.05$ )
<i>Agaricus. bisporus</i>	absent	absent	present	present	69 df
VOC ( $\mu\text{g g}^{-1}$ )					
Benzoic acid	0.39 (1.47)	-4.61 (0.00)	-1.36 (0.25)	-2.05 (0.13)	1.02
Benzene compounds	(5.72) (305.40)	2.15 (8.60)	3.54 (34.53)	2.49 (12.01)	1.35
Benzyl alcohol	5.89 (359.76)	4.96 (142.02)	1.49 (4.66)	1.08 (2.54)	0.71
1-Bromo decane	1.96 (7.10)	-0.50 (0.60)	1.39 (4.01)	0.56 (1.76)	0.45
1-Bromo heptane	-3.39 (0.024)	-4.61 (0.00)	1.44 (6.03)	1.40 (4.04)	0.62
Diethyl ester 2-butanedioic acid	2.16 (8.63)	0.08 (1.07)	1.28 (3.72)	0.33 (1.39)	0.54
2-Ethyl-1-hexanol	6.72 (830.0)	5.32 (204.5)	3.99 (68.9)	3.17 (24.0)	0.49
d-limonene	3.60 (36.59)	-2.82 (0.05)	2.25 (11.10)	1.90 (6.74)	0.67
Mequinol	-0.29 (0.74)	-4.61 (0.00)	-1.24 (0.34)	-1.87 (0.22)	0.59
Naphthalene compounds	3.81 (45.33)	1.40 (4.05)	2.62 (13.70)	1.62 (5.05)	1.06
2-Pentyl furan	4.16 (64.18)	-1.89 (0.14)	1.48 (4.73)	-0.89 (0.68)	0.67

TABLE IV. Effect of volatile organic compounds on the numbers of mushroom primordia > 1 mm diam and sporophores formed, and on the casing bacterial populations in axenic and non-axenic jar cultures contained within ventilated flasks. Primordia numbers are square root transformed values, with back-transformations shown in parentheses.

Volatile organic compound	Numbers per jar			Bacteria (ln cfu g <sup>-1</sup> casing)	
	Primordia	Sporophores	Total	Pseudomonads	
Control (start, axenic)	–	–	–	0	0
Control (start, non-axenic)	–	–	–	16.9	13.3
Control (axenic)	4.7	(22.0)	0.7	0	0
Control (non-axenic)	5.6	(31.4)	1.8	15.6	13.5
Ethanol	0.0	(0.0)	0.0	16.7	14.0
2-Ethyl-1-hexanol	2.1	(4.4)	0.3	18.3	14.1
Octane	4.1	(16.2)	2.1	17.3	14.2
1-Octen-3-ol	1.4	(1.9)	0.0	17.9	14.1
<i>cis</i> -3-Octen-1-ol	3.3	(11.0)	1.1	17.3	14.9
3-Octanone	4.5	(20.0)	1.8	16.1	13.3
LSD ( <i>P</i> = 0.05) 12 df	1.2		0.6	0.7	0.5







